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Light Microscopy of the Endoplasmic Reticulum-Membrane Contact Sites in Plants

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Summary

The existence of membrane contact sites (MCS) has been reported in different systems in the past decade, and their importance has been recognized by the cell biology community. Amongst all endomembrane structures, the endoplasmic reticulum (ER) plays vital roles in organizing the organelle interaction network with the plasma membrane (PM), Golgi bodies, mitochondria, plastids, endosomes and autophagosomes. A number of methods have been used to study the establishment and functions of these interactions, among them, light microscopy appears to be one of the most effective approaches. Here, we present an overview of the discovery of ER-PM contact sites, and highlight the latest developments in light microscopical-based techniques that can be used for their study.

Key words: Membrane Contact Sites, ER-PM contact sites, Endoplasmic Reticulum, Light Microscopy, Plants

95 **Introduction**

96 The endomembrane system is an elaborate network with various membrane-bound
97 organelles that associate with each other through membrane contact sites¹⁻⁴. Specific
98 proteins and lipids usually accumulate at these sites, and the function or composition
99 of each tethering organelles can be affected when the MCS is dysfunctional¹.

100 The endoplasmic reticulum (ER) is one of the most architecturally complicated
101 membrane networks in eukaryotic cells (Figure 1A). It is able to directly connect with
102 other organelles to regulate a variety of cellular activities, such as non-vesicular
103 material transportation, cell signaling, organelle dynamics and autophagy⁵⁻¹¹ (Figure
104 1B-D). The ER-PM contact sites (EPCS) are typical MCSs and are conserved across
105 eukaryotic cells¹²⁻¹⁴. In plants, these structures are organized by multiple EPCS resident
106 proteins and cytoskeletal components^{12,15} (Figure 1B), all of which contribute to the
107 function and formation of EPCS in response to multiple biotic and abiotic stress
108 responses^{7-9,16-18}.

109 **The discovery of ER-PM contact sites in plants**

110 In the early days, the close association between ER and other organelles was seen in
111 muscle cells using transmission electron microscopy (TEM)¹⁹, and further
112 ultrastructural studies in plants have also revealed that the cortical ER was associated
113 with the PM in different cell types²⁰⁻²². Thanks to the application of fluorescent
114 proteins (such as GFP) in endomembrane-related research, the clear observation of
115 the ER network in living cells became possible^{23,24}. It has been reported in numerous
116 studies that the cortical ER network moves dramatically, but small areas of the ER
117 remains persistent²⁵⁻²⁷ (Figure 1A) and these areas were called 'ER-anchor points'. The
118 molecular composition and biological function of these ER sub-domains remained
119 unclear for a long time²⁸. Only recently have proteins that localize to the EPCS been
120 identified in several independent studies^{7-10,17,18,29,30}, and now the biological relevance
121 of EPCS is becoming much clearer (Figure 1B-D).

122 **The observation of ER-PM contact sites using light microscopy**

A number of new systems and techniques have been developed for the investigation of MCSs. Among them, light microscopy is the most effective approach, especially for living cells. The ER polygonal structure changes dynamically within cells by altering its branching patterns of tubules, moving network junctions as well as transiting between cisternae and tubules^{31,32} (Figure 1A). These events can be easily observed using laser scanning confocal microscopy in combination with ER targeted markers. Techniques, such as persistency mapping and “AnalyzER”, have been developed in recent years to quantify the morphology and dynamics of the ER network^{27,33-35}. In this way, changes in ER dynamics, morphology or putative EPCS in different conditions have been quantified^{9,36}.

However, the resolution of conventional confocal microscopy can hardly reach below 200nm due to the diffraction barrier. In the early days, high-resolution bright-field ultraviolet (UV) microscopy was used to obtain images with superior contrast and improved resolution (since the wavelength of UV light is shorter than that of visible light) in order to study the behavior of the plasma membrane and the cortical ER. Hechtian strands that originate from the plasma membrane and remain closely attached to the cell wall after plasmolysis have been studied in this way³⁷⁻³⁹. Interestingly, ER remnants are often found within the Hechtian reticulum, indicating that the cortical ER network is indirectly linked to the cell wall, and we now know these linkages are achieved through EPCS^{36,40}.

With the help of more advanced microscopical techniques, higher resolutions could be achieved. For example, using the super-resolution imaging technique Photoactivated Localization Microscopy (PALM), the individual ER-PM junctions in HeLa cells became more resolved and clearly visible⁴¹. The molecular localization obtained through PALM images could then be analyzed using an integrated software platform to quantify the morphology of the ER-PM junctions, as demonstrated for HeLa cells as well as for neurons and fibroblasts^{41,42}.

The interactions between organelles and the cytoskeleton are highly dynamic and high-resolution microscopy which requires long acquisition times (e.g. PALM, STORM) is not easily used to study EPCS or any MCS in real time. Grazing Incidence Structured

153 Illumination Microscopy (GI-SIM) has been developed to overcome the limitations in
154 resolution, speed and z-depth of conventional light microscopy⁴³. By employing multi-
155 colour GI-SIM, the dynamic events of microtubules and ER network can be visualized
156 and measured precisely. New ER remodeling mechanisms were also identified by
157 studying the interactions between ER, microtubules and other organelles in different
158 animal cell types using this technology⁴³.

159 Fluorescence density mapping in combination with common total internal reflection
160 microscopy (TIRFM) is another approach for studying the morphology and dynamics
161 of EPCS structures. High-resolution can be achieved by exciting the fluorophore within
162 only a 200 nm depth below the PM, where cortical ER and EPCS are normally located.
163 Information on the size and shape of contact sites can be calculated by constructing
164 an intensity map⁴⁴. Taken together, although the resolution of any light microscopical
165 technique to date cannot achieve the size of the MCS interface (normally 10-30nm),
166 the current technologies do provide the quickest and most effective way to study
167 membrane and cytoskeleton dynamic at the EPCS. Although some of these novel
168 techniques (e.g. GI-SIM) are of significant interest to plant community, they have only
169 been tested on mammalian cells. Further modifications to imaging techniques are
170 required for plant cell imaging.

171 **Studying protein dynamics at the ER-PM contact site**

172 The dynamics of EPCS-resident proteins can be measured by using the technique of
173 Fluorescence Recovery After Photobleaching (FRAP). The recovery of EPCS resident
174 proteins is much slower than that of proteins that localize to the ER membrane⁴⁰. A
175 few studies have also indicated that monitoring EPCS protein dynamics may provide
176 useful information on long-term plant adaptive responses to long-term exposure to
177 ionic stress⁹. In order to study the nanoscale dynamics of EPCS-resident proteins,
178 super-resolution techniques combined with single-particle tracking can be used. Single
179 molecule microscopy methods are extremely powerful tools for investigating
180 concealed properties of a complex system. By using this technique, the diffusion and
181 trapping of STIM1 and Orai1 at EPCS of mammalian cells has been analyzed⁴⁵⁻⁴⁷. Similar
182 techniques are also well established in plants⁴⁸⁻⁵⁰, and their application to EPCS studies

in plants would be an interesting direction to progress.

Artificially designed markers and tethers used for labelling EPCS

A set of genetically encoded markers and tethers have been designed to investigate the function and dynamic regulation of ER-PM junctions. MAPPER (membrane-attached peripheral ER) is a commonly used marker for labelling EPCS in both animal and plants. MAPPER is comprised of a genetically engineered STIM1 domain fused to GFP at the ER lumen, and a FKBP12-rapamycin binding (FRB) domain fused with a polybasic motif at the cytosol; these two parts are connected by a transmembrane (TM) domain⁵¹. More recently, an *Arabidopsis* version of MAPPER, called MAPPER-GFP, has also been created⁹. The localization of MAPPER-GFP strongly resembles the plant EPCS marker SYT1 (synaptotagmin 1) and differentiates between luminal ER and ER-membrane markers⁹. Meanwhile, artificial ER-PM tethers have also been generated for studying the cortical ER architecture in yeast cells, namely TM-mCherry-CSS_{Ist2} and TM-mCherry-PH_{Osh3}. In these constructs, two alternative lipid binding motifs including a cortical sorting signal (CSS) from *Saccharomyces cerevisiae*, Ist2, and a pleskstrin homology (PH) domain from the *Schizosaccharomyces pombe* homolog of Osh3 were fused to the C-terminus of the TM-mCherry backbone. These were shown to successfully tether ER to PM⁵².

Proximity-dependent methods using fluorescent probes provide a useful toolbox for EPCS research

Benefitting from the general application of confocal microscopy, a number of fluorescent protein (FP)-based tools have been developed for targeting and identifying putative MCS. Examples include Bimolecular Fluorescence Complementation (BiFC)⁵³, Proximity Ligation Assay (PLA)⁵⁴ and dimerization-dependent Fluorescent Proteins assay (ddFP)⁵⁵. All of these techniques have been designed on the basis that signals are generated as a consequence of the close distance between target proteins and they have been used in detecting protein-protein interactions at MCS.

In the BiFC system, FP is divided into FP-N and FP-C and fused to the N- and C- termini of target proteins, respectively. A bright fluorescence will be generated only when the

target proteins interact with each other and get close enough. For example, Tao et al. (2019) employed BiFC to produce artificial ER-PM tethers in plants, and they proposed that integral membrane proteins and phosphoinositide-binding proteins contributed to ER-PM tethering. Similarly, ddFP technology involves the reversible binding of two nonfluorescent FP monomers to form a fluorescent dimer, which relies on the increased proximity or effective concentration of monomers caused by FP-FP interaction. ddFP has been successfully applied to confirm ER-mitochondria juxtapositioning in living mouse embryonic fibroblasts by targeting one half of GFP to the ER surface and the other half to the outer mitochondrial membrane (OMM). In this way, it reduces operator bias in the tethering measurements based on EM or confocal image analysis⁵⁶. Although both BiFC and ddFP are highly sensitive, care should be taken when using these techniques. Both may raise the risk of altering cellular structures and membrane organization when they are being used to study membrane-membrane interactions⁵⁷, especially when the constructs are highly over-expressed.

To overcome this potential problem of over-expression artifacts, PLA has been applied in the study of MCS localized proteins at the endogenous level and with high specificity and sensitivity in human carcinoma cell lines⁵⁸. To do this, target proteins are probed with antibodies conjugated to complementary oligonucleotide extensions. The close proximity results in a rolling-circle amplification of the signal, which can then be detected by hybridized fluorophore-labeled oligonucleotide probes⁵⁸. Recently, PLA has been used to visualize and quantify endogenous ER-mitochondria interactions between the outer mitochondrial membrane protein VDAC1 (voltage-dependent anion channel) and the ER membrane protein IP3R1 (inositol 1,4,5-triphosphate receptor) in hepatocarcinoma cell lines⁵⁹. A similar method could be applied in EPCS studies although PLA can be only used in fixed cells which is one of its main limitations.

Photo-inducible and photo-convertible markers for MCS research

It is worth mentioning that photo-inducible/convertible technologies could also contribute to MCS research. EosFP encodes a fluorescent protein that originally comes from the stony coral *Lobophyllia hemprichii*⁵⁹. It displays a green-to-red fluorescence conversion when exposed to near-UV irradiation (≈ 390 nm)⁶⁰. EosFP has been used

successfully in plants to identify putative MCS between plastids, which have been labelled with EosFP fusion proteins. Live-cell imaging has demonstrated that plastids are interlinked by tubular membranes. However, these direct connections do not appear to be transferring macromolecules as the EosFP signal could not be transferred between the interlinked plastids⁶¹.

Another optogenetic tool called LiMETER (Light-inducible Membrane-Tethered peripheral ER) has been designed to specifically label cortical ER. It contains an ER-lumen-localized fragment fused to GFP, followed by a light-sensitive LOV2 domain (Light, Oxygen or Voltage-sensing domain), and a PM-targeting polybasic tail at the C-terminus⁶²; upon blue light stimulation, the polybasic domain becomes exposed, and the protein is able to bind to plasma membranes in a close proximity⁶². The application of these photo-sensitive markers could be extended to plant cells in order to visualize the formation and dynamic changes of EPCS in a controlled manner.

Future Perspective

Professor Chris Hawes was one of the pioneers using modern light microscopy techniques for plant endomembrane research. Before the term of membrane contact site was known by the plant cell biology community, he has proposed the existence of direct interactions between ER and Golgi⁶³⁻⁶⁴ and identified ER-PM anchor sites in plants²⁶⁻²⁷. His contribution will be remembered by the plant cell biology society around the world. No doubt, the identification of MCS is revolutionary in the cell biology field. Significant progress has been made in characterizing the molecular composition of MCS in plants, as well as identifying new MCS between different structures. Although electron microscopy-related techniques (e.g TEM immunogold labelling) provide the most direct evidence to confirm the protein composition of MCS, successful application of light microscopical techniques can certainly promote our understandings in this field.

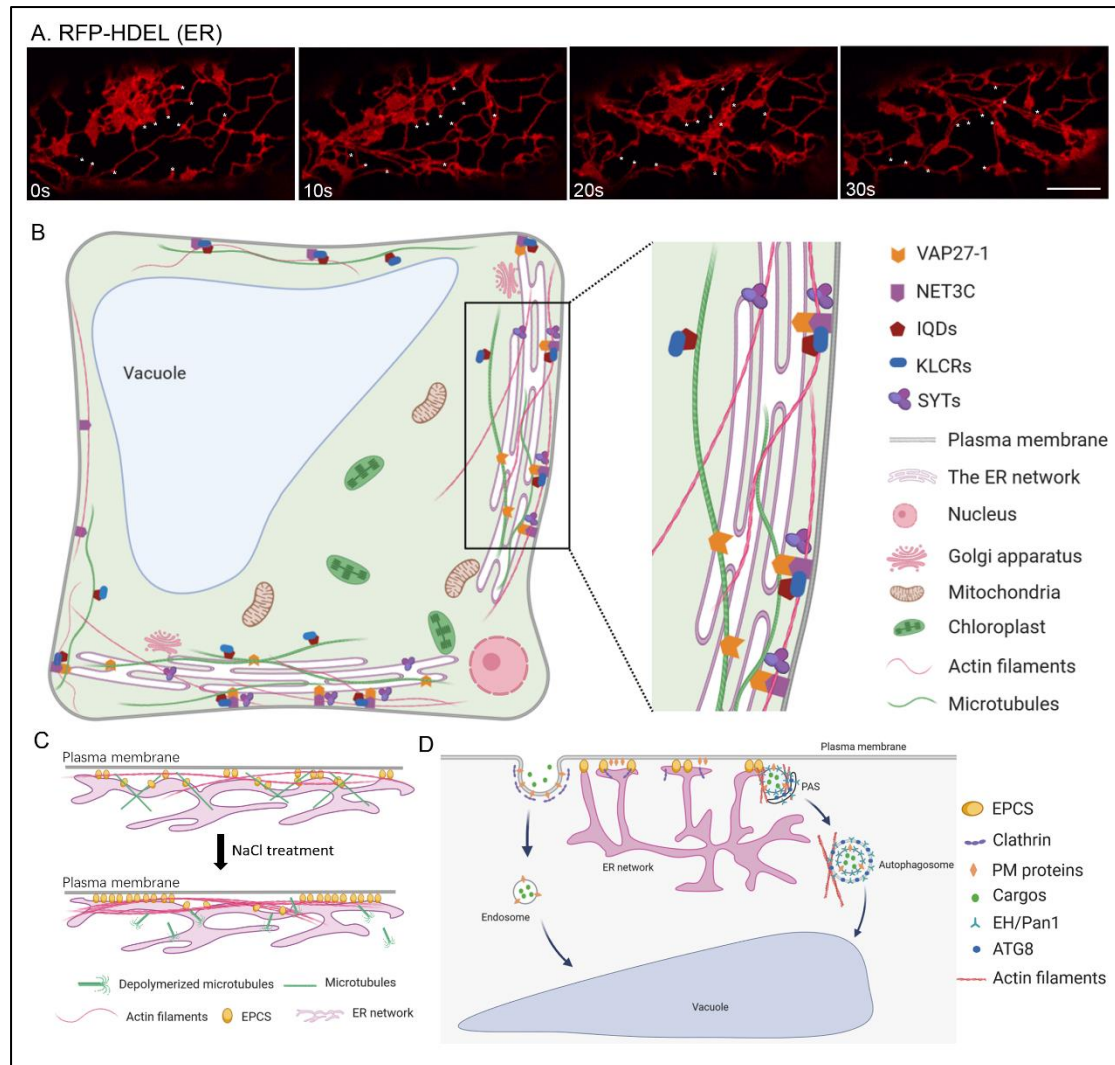


Figure 1. ER network and ER-PM contact sites in plants. **A.** The endoplasmic reticulum (labeled by RFP-HDEL) moves dramatically in plants cells, with persistent sites that connect to the plasma membrane. **B.** Diagram illustrations of EPCS-associated proteins that have been identified to date. These include ER-integral membrane proteins of the VAP27 and SYT family; an actin cytoskeleton-associated protein, Networked 3C (NET3C) and microtubule-associated proteins of the IQ67 domain (IQD) and kinesin light chain-related protein (KLCR) family^{18,29}. **C.** The attachment between ER and PM is enhanced when plants are under ionic stresses, a process that is regulated by protein-phospholipids interactions at the EPCS⁹. **D.** Known functions of EPCS in plants, such as regulation of endocytosis and autophagosome biogenesis^{11,17} (scale bar = 10µm).

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